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# DNA damage and tissue repair: What we can learn from planaria

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### ABSTRACT

Faithful renewal of aging and damaged tissues is central to organismal lifespan. Stem cells (SCs) generate the cellular progeny that replenish adult tissues across the body but this task becomes increasingly compromised over time. The age related decline in SC-mediated tissue maintenance is a multifactorial event that commonly affects genome integrity. The presence of DNA damage in SCs that are under continuous demand to divide poses a great risk for age-related disorders such as cancer. However, performing analysis of SCs with genomic instability and the DNA damage response during tissue renewal present significant challenges. Here we introduce an alternative experimental system based on the planaria flatworm *Schmidtea mediterranea* to address at the organismal level studies intersecting SC-mediated tissue renewal in the presence of genomic instability. Planaria have abundant SCs (neoblasts) that maintain high rates of cellular turnover and a variety of molecular tools have been developed to induce DNA damage and dissect how neoblasts respond to this stressor. *S. mediterranea* displays high evolutionary conservation of DNA repair mechanisms and signaling pathways regulating adult SCs. We describe genetically induced-DNA damage models and highlight body-wide signals affecting cellular decisions such as survival, proliferation, and death in the presence of genomic instability. We also discuss transcriptomic changes in the DNA damage response during injury repair and propose DNA repair as key component of tissue regeneration. Additional studies using planaria will provide insights about mechanisms regulating survival and growth of cells with DNA damage during tissue renewal and regeneration.

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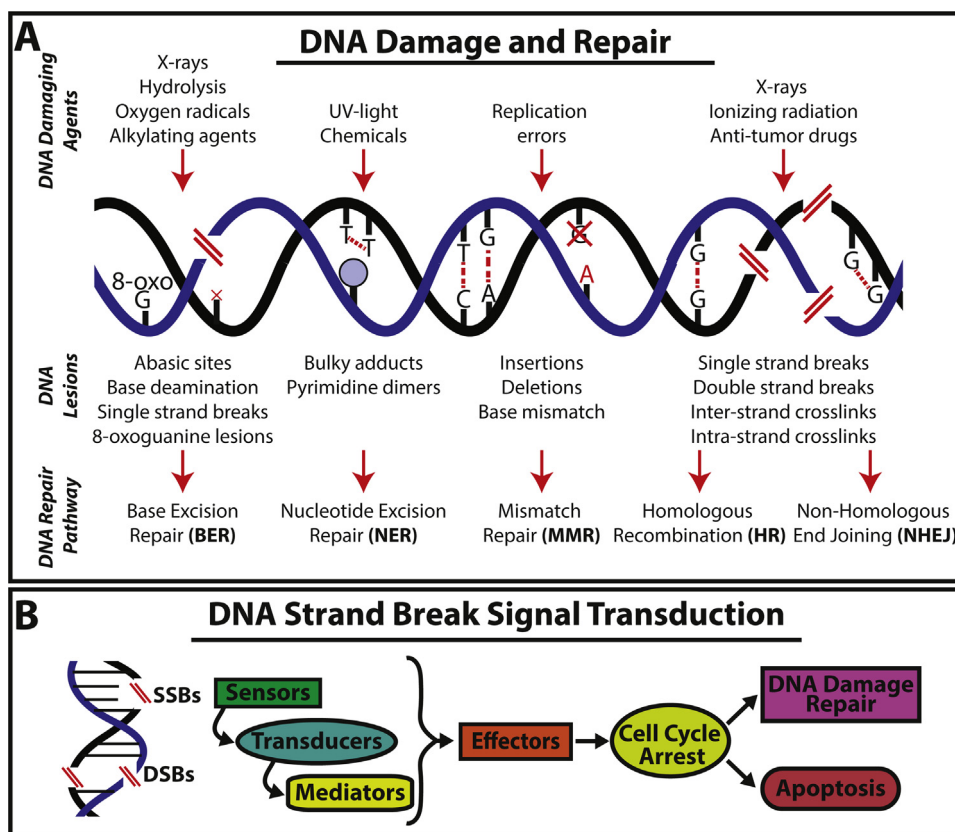
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### 1. Introduction

Preserving genomic integrity is essential to life. However, DNA is under constant threat from multiple sources, which include errors during DNA replication, products of intrinsic cellular reactions (e.g. reactive oxygen species) and environmental factors such as UV

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**Fig. 1.** DNA damage responses and stranded break signal transduction. (A) The illustration summarizes different types of DNA damaging agents, the genomic lesion they produce and the specialized DNA repair pathway deployed. For example, exposure to ionizing radiation may lead to DNA single and/or double-stranded breaks and crosslinks that can be repaired through HR or NHEJ (B) Different molecules mediate the repair of DNA strand breaks. Commonly, a signal transduction cascade involving sensors, transducers, mediators, and effector molecules that together influence cellular fate decisions to repair the damage or undergo programmed cell death.

radiation, chemical exposure, etc. (Fig. 1A) [1–5]. In humans, for example, these persistent insults generate about  $10^5$  DNA lesions per cell every 24 h [6–8]. If left unchecked, DNA damage can be transmitted to cellular progeny and potentially compromise tissue integrity and function [3,6,9–14]. Indeed, about 90% of cancer-related deaths worldwide originate from abnormalities in tissues that are constantly renewed by stem cells (SCs) [5,15–17]. Genomic instability (i.e. higher rate of genomic changes per cell division) is a major trait in almost all cancers, but the basic mechanisms regulating survival and growth of cells with DNA damage during tissue renewal remain a puzzling biomedical problem.

Exposure to DNA damaging agents generally lead to lesions that are common to all living organisms (Fig. 1A) [6,18,19]. Thus, highly conserved mechanisms of DNA repair have evolved to preserve genetic information and proper cellular function [19–23]. DNA damage response (DDR) sensors and effectors are continuously deployed and are mediated by a specific set of proteins with the goal of re-establishing genomic integrity (Fig. 1B) [19]. Importantly, the timely deployment of DDR is synchronized with cellular responses leading to critical decisions that may involve cell cycle arrest, apoptosis, senescence and DNA repair (Fig. 1B) [18,24]. Altogether, the varying responses to DNA damage aim at preventing exhaustion and abnormal transformation of SC pools while maintaining their ability to mediate tissue homeostasis.

The process of DNA damage and its cellular response have been widely documented by *in vitro* studies and organ specific experimental models. However, the field has benefited less by simultaneous analysis of DNA damage and SC-mediated tissue renewal at the organismal level. We believe that studies merg-

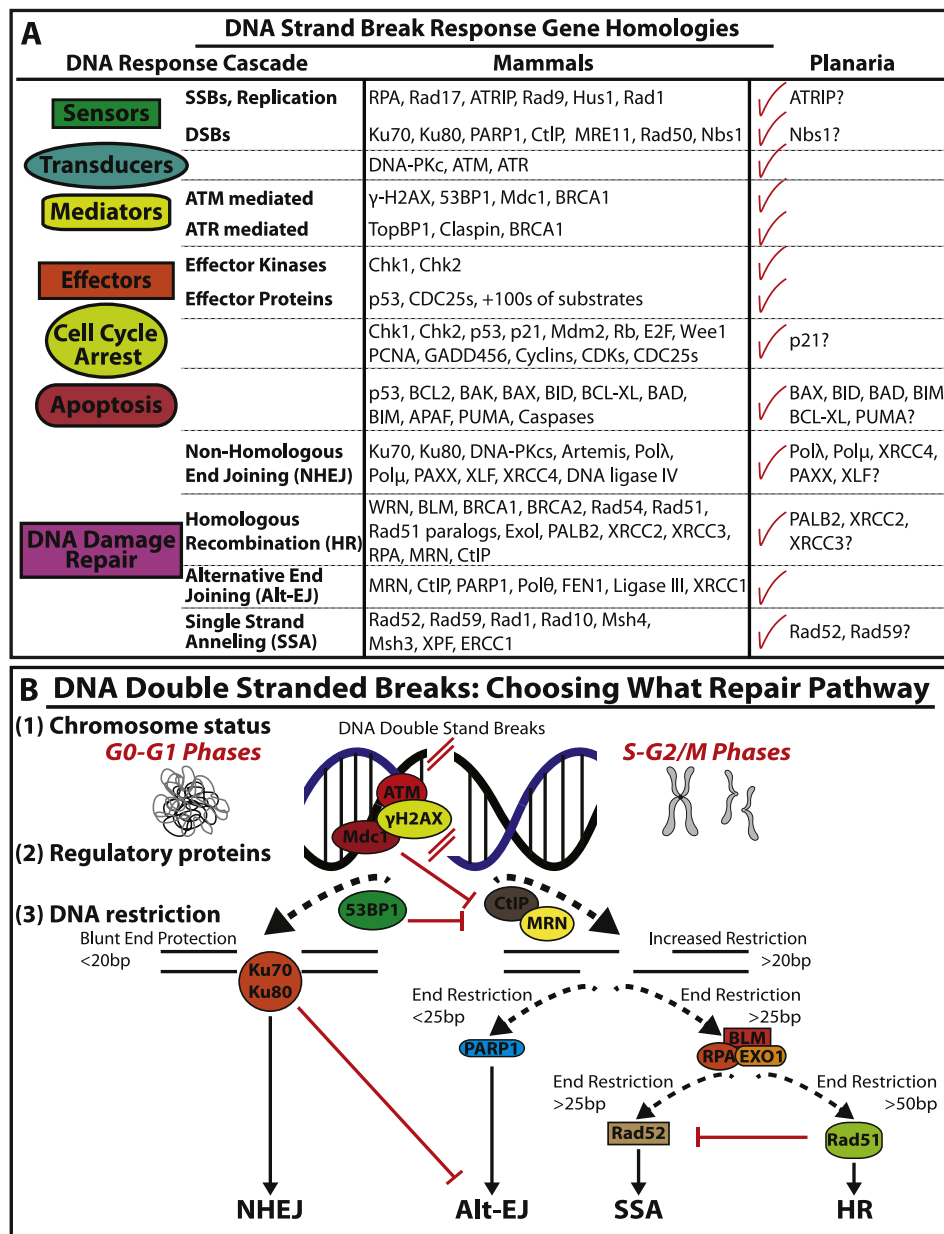
ing the cellular response to DNA damage, while attending body demands of cellular turnover may bring important insights about intercellular crosstalk that affects cellular fate decisions in the adult body. For example, there are patterns of regional differences of cell proliferation along the anteroposterior (AP) body axis that affect the fate of SCs and their progeny during tissue renewal, regeneration and carcinogenesis [25–31]. These regional differences are evolutionarily conserved across different species and recent studies have shown that the fate of cells with DNA damage are susceptible to regional signals [22,23]. In an attempt to complement studies of DDR/DNA repair during tissue renewal and cancer formation, we introduce a simplified model system represented by the planaria flatworm. Planaria possess high rates of cellular turnover and tissue regeneration is driven by adult SCs called neoblasts, which facilitate studies about SC-mediated tissue renewal and DNA damage [32–37]. We present a brief description of the DNA lesions and the molecules involved in repair with special emphasis on double-strand breaks (DSBs), the most dangerous form of DNA damage [8,20,38,39]. We highlight the role of DNA damage during tissue renewal and its possible impact in aging and discuss how recent experimental planaria models associated with DNA damage may provide insights about the SC response during adult tissue maintenance and regeneration. In addition, we identify through data mining of various transcriptomic datasets that the DDR is a critical component of the large-scale tissue homeostasis and regeneration in planaria. Ultimately, we propose the use of planaria as a convenient model to address evolutionarily conserved mechanisms of DDR and DNA repair during tissue repair and regeneration in the adult body.

## 2. Tissue renewal and stem cell response to DNA damage

Organismal lifespan relies on faithful renewal of aging and damaged tissues [40–46]. SCs generate cellular progeny to maintain adult tissues and in humans, this is a daunting process that requires daily demand of billions of cells that could span over a century [47–50]. Tissue renewal is extremely complex, fulfilling different dynamics of cellular turnover that appear unsynchronized among tissues. For example, the small intestinal epithelium is renewed in about 5 days, while epidermal cells in the skin are replaced every 10–30 days, let alone cells within blood tissue have different renewal rates from 1 day to several months [51–54]. Despite

its relevance to physiology and disease, it remains poorly understood how this large scale renewal process is coordinated and how it becomes liable as organisms grow older.

Both tissue maintenance and the capacity to preserve genomic integrity decline with age. Intriguingly, there is positive correlation between DNA repair and lifespan in a variety of organisms [40,41,55,56]. For example, longer-lived species such as humans and naked mole rats consistently display higher expression of genes associated with DNA repair, which supports the idea that preservation of genomic integrity is paramount to the longevity of an organism [41]. The mechanistic process by which genome integrity declines with age is not well understood. Nonetheless, several lines



**Fig. 2.** Planaria display evolutionary conservation of the mechanisms involved in DNA double strand break (DSB). (A) Gene homologies for sequences regulating DDR and signal transduction mechanisms are found in planaria. For simplicity the attention is mostly focused on mediators of DSB. Checkmarks indicate conservation between the planarian species *S. mediterranea* and humans. However, some genes remain unfound in the planarian genome indicated by a question mark. (B) DSB repair is mediated by four different pathways (e.g. NHEJ, Alt-EJ, SSA and HR) however the choice of these pathways are determined by three mechanisms. (1) The status of chromosome homology which is dependent on what phases of the cell cycle DSBs occur (e.g. homology during S-G2-M phases which favors HR). (2) The abundance of regulatory proteins in each cell cycle phase. For example, increased expression of ATM, Mdc1 and 53BP1 have been shown to favor NHEJ in the G0/G1 phase of the cell cycle by suppressing MRN and CtIP expression needed for HR-mediated repair. (3) Lastly, the restriction of DNA blunt ends. The length of DSB nucleotide base pairs give rise to blunt end protection or a series of end restrictions. (e.g. <20 bp for NHEJ and >50 bp for HR mediated repair).

of evidence imply continued exposure of SCs to DNA damage plays a major role in age-related dysfunctions such as cancer and degenerative diseases [1,3,9,16,57–62]. Eventually, unrepaired genetic lesions may result in SC attrition, cellular transformation and aberrant differentiation that could lead to defective tissue renewal [1,3,9,10,61–63]. It is unclear whether the increased genome alterations are due to individual or combined effects of: (i) impaired DDR, (ii) increased levels of DNA insults, (iii) epigenetic modifications and telomere shortening with age, (iv) higher susceptibility to damaging agents in SC and progenitor populations (e.g. quiescent, cycling), and/or (v) defects in cell fate decision mechanisms upon demands of cellular turnover. In reality, this list could be more extensive when cellular turnover is considered in the complexity of the whole organism. Systemic factors associated with inflammation, oxidative stress, metabolism, etc., which also have inputs on decisions of cellular proliferation and apoptosis during tissue turnover [1,3,10,61,63–67]. Collectively, DNA damage greatly influences the ultimate fate of the cell. Nevertheless, it is less clear how cellular decisions are prioritized when physiological demands of tissue turnover are in play and how cancer and degenerative diseases evolve from defects in these cellular decisions.

Increasing evidence demonstrates that preservation of genomic integrity and systemic reduction in DNA damage could be enhanced by physiological or pharmacological manipulations. Specifically, treatments aimed at replenishing the coenzyme NAD<sup>+</sup> (nicotinamide adenine dinucleotide) appear to reduce the decline in adult tissue maintenance with age by enhancing DNA repair in animal models, which altogether lead to improvement in lifespan and healthspan [68–70]. These results strongly suggest that it is possible to alter the fate of both DNA repair and cellular turnover with therapeutic interventions. However, many questions still remain and additional model organisms are needed to simultaneously analyze and integrate process of tissue renewal and DNA repair in the complexity of the whole organism. We believe the planaria model system could provide important insights in this regard.

### 3. Planaria as a model to study DNA damage and tissue renewal

Planaria are members of the phylum Platyhelminthes (flatworms) and are classically known for their robust regenerative capabilities [71]. Planaria display constant cell renewal and undergo repair upon injury to their tissues and organs (e.g. digestive, nervous, muscle, etc.) [34]. The planaria *Schmidtea mediterranea*, which is the most common species used worldwide to study aspects of tissue homeostasis, contains a large pool of SCs called neoblasts. Neoblasts are recognized as the only cells with capacity to proliferate in *S. mediterranea* and therefore, serve as the sole source of new cells that support the dozens of different tissues types [32,34,71–73]. The neoblast diversity is only beginning to be elucidated and so far, four subpopulations have been described (e.g. Sigma, Gamma, Zeta and Nu) that display restricted potential to generate and maintain tissues [72,74,75]. This diversity within planaria neoblasts allows for the integration of local and environmental stimuli throughout its lifespan to maintain tissue homeostasis.

Similar to SCs in other organisms, neoblasts are in constant crosstalk with their surroundings and are influenced by local and systemic signals involving metabolic status, neural inputs, tissue integrity, etc. In the presence of nutrients, planaria increase the body size by incorporation of new cells. Conversely, starvation conditions lead to reduction in animal size by elimination of cells that maintain body proportion [76–78]. Neoblasts also sense and respond to tissue injury by mounting a multi-step proliferative

response that mediates the regrowth of missing and damaged parts [79–81].

The capacity to regulate SC division in response to physiological demands and injury has been attributed to the conserved tumor suppressors and oncogenes within the planaria (e.g. PTEN, AKT, p53, Rb, SMG) [82–87]. Planaria rarely develop cancer but can be forced to undergo cellular transformation after treatment with carcinogenic compounds or manipulation of tumor suppressor genes [83,87–90]. In line with previously described organisms, preservation of DNA integrity is paramount for tissue homeostasis and extended lifespan in planaria. Recent interest in dissecting mechanisms of DNA repair in planaria revealed the evolutionary conservation of key regulators such as *Rad51*, *p53*, *Rb*, *Ubc9*, *Brca2*, and *Rad54B* that are activated in response to endogenous and exogenous environmental insults [22,23,84,91–93]. Functional studies of DNA repair pathways identified patterns of SC exhaustion and tissue renewal defects similar to those observed in mammals [10]. These planaria features confer unique advantages to analyze critical parameters in response to DNA damage in the context of the whole body. Thus, we propose the use of *S. mediterranea* as a simplified platform to address cell fate decisions in the presence of genomic instability during large-scale SC-mediated cellular turnover and tissue repair.

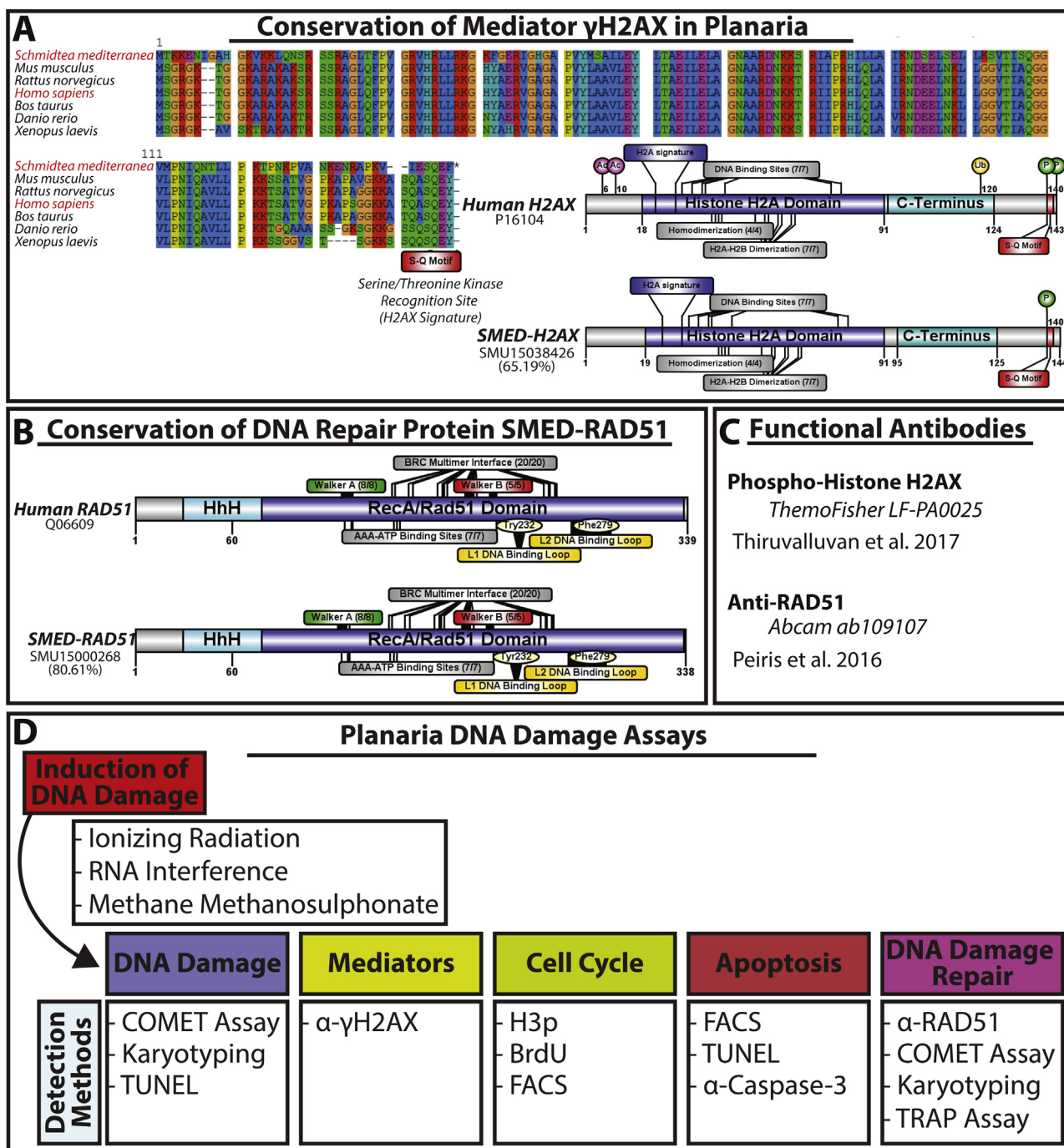
### 4. Evolutionarily conserved DNA damage repair mechanisms exist in planaria

Older specimens of *S. mediterranea* are phenotypically indistinguishable from younger ones, which highlight the efficient mechanisms of unlimited cellular renewal in planaria [34]. This also implies that planaria contain efficient DNA repair mechanisms to combat endogenous and exogenous insults that normally deplete SCs in adult tissues, thus preventing aging and cancer-like phenotypes [88]. Indeed, high-throughput query on genomic resources [94,95] have allowed us to identify a wide range of DSB recognition and repair homologs in planaria (Fig. 2A). Specifically, we uncovered components of signaling pathways involved in DNA damage recognition, signaling transduction and effector outcome (e.g. cell cycle arrest, cell death and DNA repair).

Though DNA damage can affect cells in numerous ways, DSBs represent the most severe form of DNA damage as they occur when both strands of the DNA double helix are broken in close proximity. Two DSBs within a cell are capable of forming chromosomal translocations and some estimates establish that 10–50 DSBs occur per cell per cycle [8,39,96,97]. Generally, DSBs can be repaired through: non-homologous end joining (NHEJ), homologous recombination (HR) and their alternative pathways: alternative end joining (alt-EJ) and single strand annealing (SSA) (Fig. 2B). The selection of DSB repair pathways is determined by three independent variables: cell cycle phase (e.g. chromosome status), the abundance of regulatory proteins in each cell cycle phase and the resection of DNA blunt ends (Fig. 2B). These mechanisms of DSBs repair have been extensively reviewed elsewhere [3,5,20,97–100].

Detailed evaluation of the planaria genome revealed an important molecular conservation of the DDR mediator  $\gamma$ -H2AX and DSB repair protein RAD51 (e.g. ~65% and ~81%, respectively) (Fig. 3A, B). Further analysis also showed the presence of key signatures of their phosphorylation sites (e.g. S-Q motif the signature for  $\gamma$ -H2AX) or binding domain activity (e.g. Rad51's Walker A/B, L1/2 and BRC domains) (Fig. 3A, B). The molecular conservation of these molecules in planaria also facilitates the possibility of using commercial antibodies to evaluate the spatial distribution of DDR proteins at cellular and organismal levels (Fig. 3C) [22,23,101]. DSBs can be induced in planaria through ionizing radiation (IR), RNA

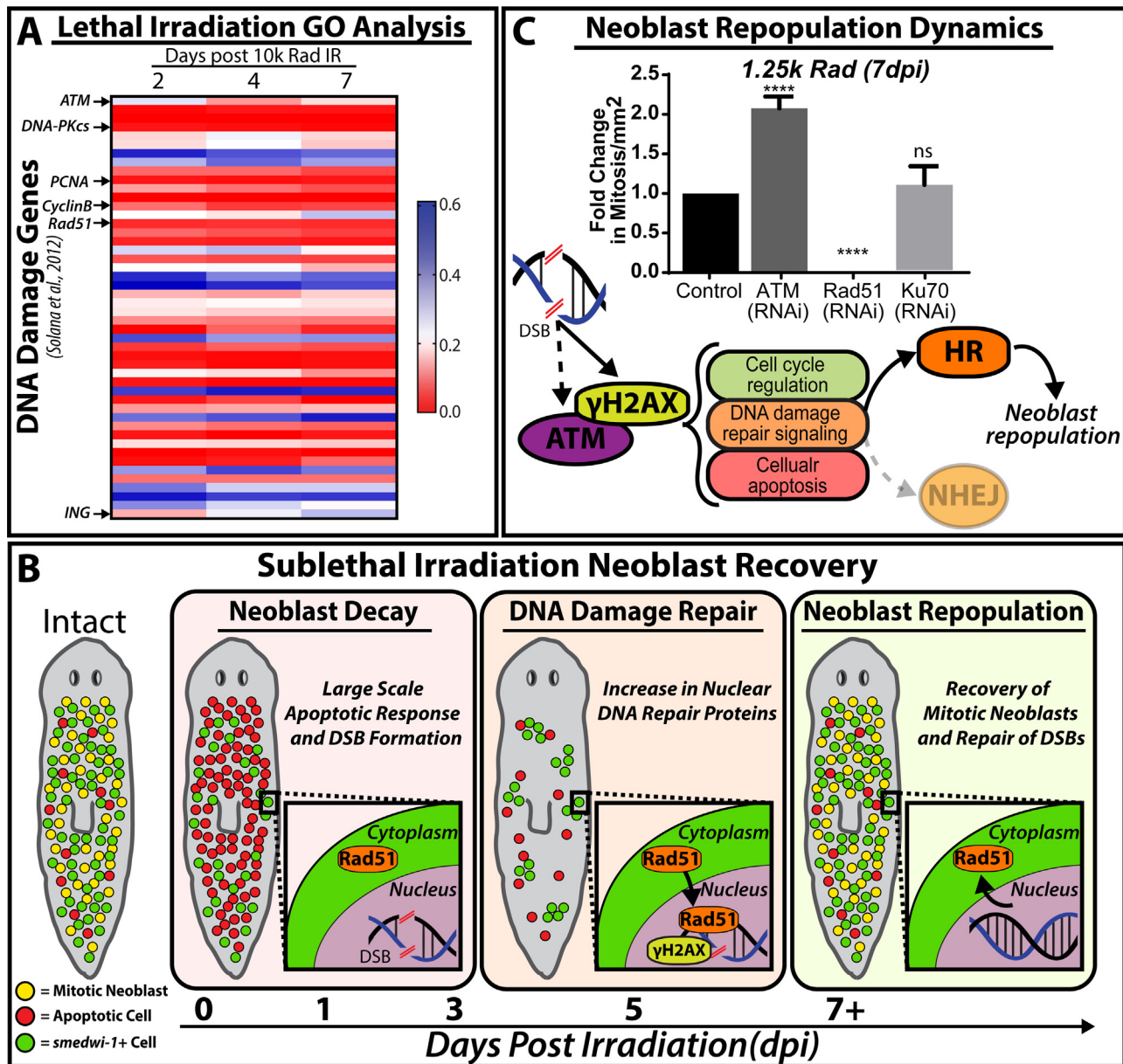




**Fig. 3.** Molecular conservation of key proteins regulating DNA double strand break repair and DNA damage assays in planaria. (A) Protein alignment of planaria  $\gamma$ H2AX and homologs indicate conservation of  $\gamma$ H2AX's S-Q motif among species. The *S. mediterranea* mediator  $\gamma$ H2AX scores 65.19% similar to the human homolog. Sequence alignment was obtained by CLUSTALW. Using Illustrator for biological sequences (IBS version 1.0) we were able to create diagrams for both human and SMED- $\gamma$ H2AX protein sequences. Human protein length is 143 amino acids (aa) versus 144aa in SMED- $\gamma$ H2AX. Within this protein both the planaria and human sequences contain Histone H2A domain (dark blue, 73aa long) and a C-terminus (light blue, 30aa long). Within the Histone H2A domain both contain the H2A signature (6aa long), DNA binding sites (7/7 sites) homodimerization sites (4/4 sites) and H2A-H2B dimerization sites (7/7 sites). Importantly, both contain the S-Q motif (red domain) phosphorylation on Ser-139 site in the S-Q motif. However, Human  $\gamma$ H2AX contains acetylation sites (6aa and 10aa), ubiquitination site at 120aa and another phosphorylation site at 143aa. (B) The *S. mediterranea* RAD51 sequence shares 80.61% similarity with the human homolog and contains all the documented characteristic binding domains. Both contain a helix-hairpin-helix (HhH) motif (light blue, 48aa long) with a large RecA/Rad51 domain (dark blue, 277aa long). Within the RecA/Rad51 domain there are two key features Walker A (green and 8/8 sites) and Walker B (red, 5/5 sites) binding regions. Other features of the protein are the BRC multimer interface (20/20 sites), (AAA-ATP binding sites (7/7 sites), L1/L2 DNA binding loops (yellow) which contain a Tyr 232 and Phe 279, respectively. (C) Details of commercial antibodies that recognize the *S. mediterranea* RAD51 and  $\gamma$ H2AX homologs. SMED-RAD51 antibody generated by the Sánchez-Alvarado/Hawley labs (Xiang et al. [92]) recognizes similar peptide size as the commercial human RAD51 (Thiruvalluvan et al. [23]). (D) General assays and detection methods for inducing and quantifying the response and outcome of DNA damage in native stem cells in the planarian model.

interference (RNAi) and through drug exposure such as methyl methanesulfonate (MMS) [22,23,90]. In order to gauge the effects of these DSB inducing strategies, three key strategies have been optimized (e.g. TUNEL, COMET assay and karyotyping) [22,23]. The

COMET assay, under alkaline conditions (pH > 13), is commonly used to monitor DNA integrity with focus on DSBs. In addition, karyotyping protocols have been optimized to assay abnormalities found in neoblast-specific chromosomes [22,102]. FACS protocols



**Fig. 4.** Neoblast response and repopulation post irradiation is attributed to functional DNA damage detection and repair. (A) Lethal irradiation of planaria (10k rad) irreversibly depletes neoblast populations. Upon analysis of GO term annotations published by Solana et al. [111], genes involved in DDR and repair along a 7 day time course were selected. The heat map shows changes in gene expression at different times post-irradiation with color red indicating reduction and blue increase in gene expression (scale bar to the right). DNA damage response triggers upregulation of ATM gene expression accompanied by a decrease in cell cycle and DNA repair gene expression (e.g. PCNA, CyclinB and Rad51, respectively). Note that the inhibitor of growth (ING) gene expression is upregulated after 2 hpi. (B) Cartoon depiction of neoblast response to sublethal irradiation. Upon sublethal irradiation, 1–3 dpi, neoblast decay arises from a large scale apoptotic response and a lack of RAD51 nuclear translocation accompanies increases of DSBs. Secondly, the remainder of *smedwi-1+* neoblast clusters begin to slowly expand and cells exhibit a peak of DDR and DNA repair proteins at 5dpi (e.g.  $\gamma$ H2AX and RAD51, respectively); marking the DNA damage repair response. Lastly, neoblast repopulation occurs 7+ dpi with increases of *smedwi-1+* cells, recovery of mitotic neoblasts and a decrease in DSBs. Cellular events are depicted by the following: mitotic neoblasts (yellow), apoptotic cells (red) and *smedwi-1+* cells (green) (C) Fold change in mitosis in RNAi and mock control animals is quantified 7dpi after 1.25k rad (sub-lethal dose). Notice the increase in mitotic events upon *ATM*(RNAi) and the inability for neoblasts to recover post *Rad51*(RNAi). Underneath, proposed mechanism of neoblast repopulation post sublethal IR show a possible role of *Smed-ATM* functioning as a transducer of DSB signal in tandem with  $\gamma$ H2AX phosphorylation. Further, this model implies that HR signaling is a key player in neoblast repopulation as RNAi of NHEJ did not affect neoblast repopulation. Dotted arrows and shaded colors imply uncertainties within the model and further experiments are required to validate these assumptions and interactions. \*\*\*\* $p < 0.0001$ ; one-way-ANOVA.

have been implemented to monitor neoblast populations, cell cycle progression and cellular apoptosis [103–106] (Fig. 3D).

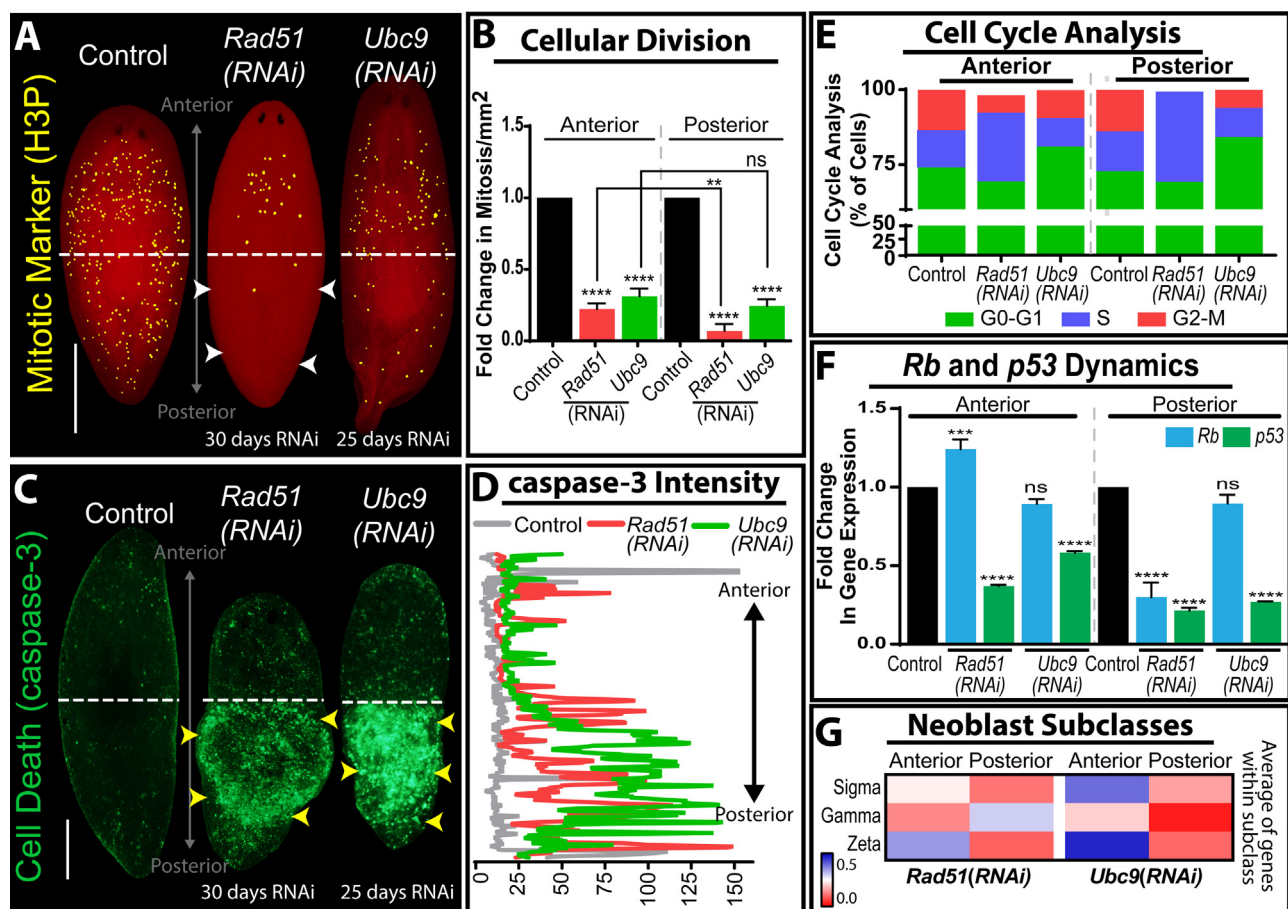
Planaria tolerate relatively high doses of IR, far surpassing the thresholds of exposure that are known to be lethal in mammals [107,108]. Thus, the DDR in planaria can be analyzed by exposing animals to IR. For example, exposing planaria to IR above 3000 rad irreversibly eliminates neoblasts, abolishes regeneration, and leads to animal death in about three weeks [37,71,91,109–111]. Lethal

doses of IR has been traditionally used as a tool to identify neoblast-associated markers and mechanisms of neoblast repopulation during tissue transplantation and irradiation [37,71,91,109–111]. However, data mining of recent transcriptional data involving samples from a seven day time course upon lethal IR [112] revealed a persistent upregulation of genes involved in DNA damage sensing and signal transduction of DSBs throughout the time course. Interestingly, there was high expression of ATM but reduced gene

expression of DNA-PKcs, required for NHEJ, throughout the time course (Fig. 4A). Similar effects are observed in primary human fibroblasts cell lines during chronic exposure to IR [113]. Because lethal IR exposure irreversibly eliminates neoblasts, the results confirmed that key regulators of cell cycle progression and markers of proliferating neoblasts were nearly abolished upon IR (e.g. *PCNA* and *cyclinB*). Furthermore, the irreversible elimination of neoblasts may also result from a gradual increase in the expression of the inhibitor of growth protein (ING) (Fig. 4A). Members of the ING family have been found to negatively regulate EGFR/PI3K/Akt signaling pathway, which is central to planaria neoblast re-population post IR [91,114]. Thus, the increased amount of IR-induced DNA damage together with upregulation of ING expression may act together to eliminate neoblast and prevent residual cell proliferation post-IR. These findings also indicate that most components of the DDR are associated with neoblasts.

Exposure to sub-lethal doses of IR (i.e. 1000–1750 rad) leads to a partial elimination of neoblasts, which allow for studies of DDR, SC repopulation and recovery during adult tissue renewal

[91,115] (Fig. 4B). The re-establishment of mitotic activity post sub-lethal IR has been attributed to EGF signaling and active DNA repair mechanisms (e.g. Rad51 and Rad54B) [91,116]. Sub-lethal IR depletes neoblast mitotic activity within 24 h, accompanied by a significant spike in apoptosis and DSBs. There is a gradual increase in DSB repair that peaks at five days post IR as determined by RAD51 gene/protein expression and RAD51 nuclear translocation (Fig. 4B). Neoblasts uniquely express the gene *smcdwi-1* (*piwi-1*, henceforth) [37,75,117]. *piwi-1* expression is currently used as the gold standard to recognize the presence of neoblasts and their distribution. *piwi-1+* cells clusters are severely reduced during the first 7 days post-irradiation (dpi, 1250 rad) and begin to expand after 9 dpi [91,115]. However, mitotic activity is detectable after 7 dpi (Fig. 4B). Neoblast repopulation depends on EGF signaling that requires active DNA repair mediated by ATM, Rad51, Ku70 and Rad54B [91,116]. Interestingly, functional disruption of ATM with RNA-interference (RNAi) leads to an accelerated re-establishment of mitotic activity 7dpi [116] (Fig. 4C). ATM is an important player of the DDR that influences cellular decisions upon IR through reg-



**Fig. 5.** *Rad51* and *Ubc9* inhibition in planaria yields high levels of DSB and region-specific neoblast responses (A) Whole mount immunostaining of mitotic neoblasts marked by Histone3 phosphorylated (H3P) in mock control, *Rad51*(RNAi) and *Ubc9*(RNAi) models. Note the difference in mitotic cells along the anteroposterior axis in the *Rad51*(RNAi) group; white arrow heads indicate a severe decrease in mitosis in the posterior versus the anterior. (B) Levels of mitotic cells across the anteroposterior axis, confirms the asymmetric distribution of mitoses is specific to *Rad51*(RNAi) but not to animals subjected to *Ubc9*(RNAi). (C) Whole mount immunostaining of cell death marked by the caspase-3 antibody. Note the increase of apoptotic events (green signal) is concentrated in the posterior region of both *Rad51*(RNAi) and *Ubc9*(RNAi) animals (yellow arrowheads). (D) Quantification of the fluorescent intensity signal from caspase-3 stained *Rad51*(RNAi) and *Ubc9*(RNAi) animals confirming the increased cell death in the posterior regions compared to the anterior and control (yellow arrowheads coincide with the increase in cell death). Intensity readings were obtained by tracing a line in the middle of the animal from the anterior to the posterior region using ImageJ software. (E) Cell cycle analysis using flow cytometry in cells dissociated from the anterior and posterior regions in animals subjected to *Rad51*(RNAi) and *Ubc9*(RNAi). Different phases of the cell cycle are color coded and show that *Rad51*(RNAi) and *Ubc9*(RNAi) animals display different effects on cell cycle represented by important reduction in M-phase in the posterior region of *Rad51*(RNAi), while *Ubc9*(RNAi) animals tend to halt cell cycle in G0/G1 phases but some cells still continue to divide. (F) Fold change in gene expression of *Rb* and *p53* relative to the control. *Rb* expression is asymmetrically upregulated in the anterior of *Rad51*(RNAi) but it does not appear affected in the same manner after *Ubc9*(RNAi). (G) Heat map, representing fold change in the average of gene expression of neoblast subclass (sigma, gamma, zeta) markers in the anterior and posterior regions. Red indicates diminished gene expression and blue increased gene expression. \*\*p < 0.01; \*\*\*p < 0.001; \*\*\*\*p < 0.0001; two-way-ANOVA. Scale bars: 200  $\mu$ m.



ulation of p53/p21 axis to facilitate cell cycle checkpoint arrest [113,118]. Thus, we can postulate that *Smed-ATM* may be key to facilitating an appropriate cellular response to DSBs (e.g. detection and cell cycle arrest). Further experiments will be required to identify the role of *Smed-ATM* upon IR in cell cycle regulation and determine if *Smed-ATM(RNAi)* hyper-proliferative neoblasts are genomically stable; altogether validating the conservation of this protein in planaria. The interplay of DNA repair and neoblast repopulation is only beginning to be understood but the recent evidence suggest that mechanisms of HR are the predominant repair pathway in planaria [23,91,116]. This is further supported by results demonstrating that RNAi of *Rad51* and *Rad54B* in sub-lethally irradiated animals fail to repopulate *piwi-1+* cells and mitotic activity, resulting in lethality. Conversely, dynamics of mitotic repopulation in *Ku70(RNAi)* sub-lethally irradiated animals are indistinguishable from untreated control group [22] (Fig. 4C). All together, these results imply that *Smed-ATM* is a key upstream regulator of cell fate in response to IR-induced DSBs and HR is the dominant pathway used in repairing damaged DNA in planaria.

## 5. Genetic models of DNA damage in *S. mediterranea*

Two independent DNA damage models have been developed in planaria by disrupting gene function via RNAi of *Rad51* and *Ubc9* [22,23]. Planaria homologs of *Rad51* and *Ubc9* show high evolutionary conservation with higher organisms and their disturbance revealed important patterns of regional defects along the AP axis (Fig. 5A–D). Furthermore, RNAi of *Rad51* and *Ubc9* display loss of genomic integrity, specifically by the accumulation of DSB throughout the planaria body [22,23].

*Rad51* is required for nucleofilament formation and without functional RAD51 protein the HR repair complex cannot form [20,119,120]. Full knockout of *Rad51* results in embryonic lethality in mammals [121,122]. Nonetheless, it is possible to knockdown the HR pathway but it is challenging to evaluate organismal SC response and track their progeny in an environment of genomic instability. Thus, the planaria model system offers unique opportunities to overcome these limitations by enabling the possibility of disrupting HR while SC attend systemic demands of cellular turnover and repair. On the other hand, SUMOylation is a dynamic and reversible post-translational modification that requires the cooperation of a host of proteins [123]. Critical to this pathway is UBC9, which determines protein SUMOylation [123]. SUMO attachment regulates protein function as it can affect protein localization, stability, protein–protein interaction, cause conformational changes or act as a hub to form multi-protein complexes [124]. Mounting evidence suggests SUMOylation plays a critical role in the regulation of DSB repair [125–129]. It does so in three ways: (1) regulates protein stability, DNA binding ability and localization of sensors and effectors of DSB repair, including both NHEJ and HR; (2) leads to creation of an open chromatin state more amenable to repair by controlling epigenetic modification through modulation of various methylases and acetylases; (3) orchestrates successful DDR response by coordinating multiple types of post-translational modifications, most notably stubl-mediated ubiquitination [130–139].

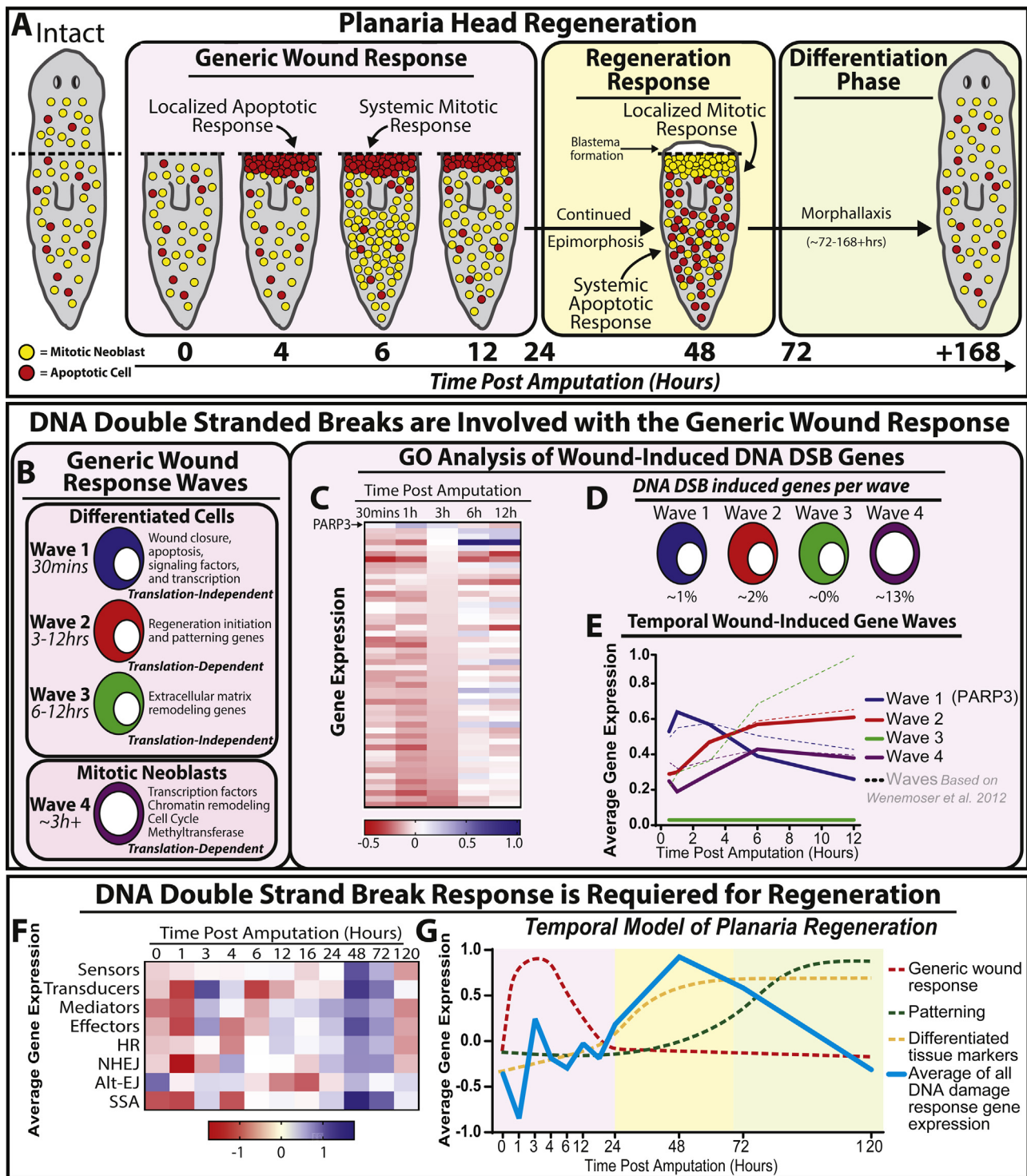
### 5.1. Functional disruption of *Rad51* and *Ubc9* affects tissue homeostasis in planaria

The unique feature of the *Rad51* and *Ubc9* knockdown models is the prevalence of DNA damage, especially DSBs that is present throughout the planaria body [22,23]. Molecular analysis in both models revealed a transient presence of DNA DSBs and chromosomal abnormalities that progressively increased over time. This is

consistent with the role *Rad51* plays in DSBs repair through HR. However, the mechanism driving this phenomenon in the *Ubc9* phenotype was less evident. Two observations were critical to relate SUMOylation to DNA damage: (i) the regional defects in animals subjected to *Ubc9(RNAi)* were similar to those observed in *Rad51* phenotype and (ii) late stages of the *Ubc9* phenotype display increase in RAD51 and  $\gamma$ -H2AX protein expression, which appeared in clusters along the AP axis. Additional analysis revealed that DNA damage in *Ubc9(RNAi)* is due to the inability of RAD51 to translocate from the cytoplasm to the nucleus to repair DSBs [23]. This finding links the two models together and additionally explains phenotypic similarities, which altogether supports the idea that HR is the prominent pathway for repair of DSBs in planaria.

The induction of DNA damage after *Ubc9* and *Rad51(RNAi)* results in a cascade of cell fate decisions led by cell cycle arrest [22,23]. Cell cycle analysis revealed that while most cells in the *Rad51(RNAi)* were arrested in S phase, cells in *Ubc9(RNAi)* animals were primarily arrested in the G1 phase (Fig. 5E). *p53* and *Rb* commonly regulate neoblast fate decisions (i.e. apoptosis, proliferation and cell cycle arrest) during tissue renewal and regeneration. This is also the case in the presence of DNA damage in planaria but intriguingly; we found that *p53* gene expression is downregulated across the AP axis in both RNAi groups (Fig. 5F). However, there were stark differences in *Rb* expression. Specifically, there is an increase in *Rb* expression in the anterior region of *Rad51(RNAi)* animals whereas there is no significant change in *Ubc9(RNAi)* group (Fig. 5F). Although our knowledge of *Rb* dynamics relies on gene expression data, it is tempting to link increased *Rb* expression with cell cycle arrest. Canonically, *Rb* is thought to be an important regulator of the G1/S checkpoint and studies suggest that overexpression of *Rb* can increase rates of cellular survival and predispose cells to become more cancerous [140,141]. Furthermore, the genomic instability driven cell cycle arrest in both lead to interesting changes in tissue homeostasis and cellular turnover, specifically in terms of cell survival and death [22,23]. While both models coincide in a significant decrease in the cycling neoblasts, the *Rad51* model reveals a remarkably difference across the AP axis, specifically loss of survival in the posterior region. This is likely due to the differential expression of cell fate regulators *p53* and *Rb*. On the other hand, both models show a massive increase in cell death in the tail region with significantly less cells dying in the anterior. It is possible that *Rb* is acting as switch for allowing cell survival in the anterior but not the posterior. Another explanation as derived from experimentation in the *Ubc9(RNAi)* model, where the cell death is partially attributed to attenuation of Hedgehog signaling, which is known to be an important regulator of posterior polarity in planaria. Whether the same mechanism is driving cellular decisions in the *Rad51* phenotype requires further experimentation.

A remarkable finding from these studies is that some SCs in the anterior region are able to overcome surveillance mechanisms and continue proliferating with genomic instability [22,23]. In the *Rad51(RNAi)*, this is in part due to increased expression of *Rb* and neural inputs in the anterior region. Ectopic introduction of brain tissue in the posterior region, induce neoblast proliferation with DSB. These findings highlight the possibility of intercellular effects, whereby neural signals alter fate decisions of neoblasts with DSBs. Likewise, these results also prompt future studies about possible neural regulation of *Rb* signaling that facilitate proliferation of neoblasts with DSBs. Alternatively, it is possible that a subset of neoblasts is endowed with proliferative capacity to give rise to cancer-like cells in the anterior. Multiple neoblast subtypes have been characterized [72,74,75]. We found that gene expression of markers associated with zeta neoblasts are increased in the anterior for both *Ubc9* and *Rad51(RNAi)* animals (Fig. 5G). Recent research demonstrates the intriguing possibility that inhibition of Hippo signaling trigger dedifferentiation of postmitotic progenitors in pla-



**Fig. 6.** DNA damage repair (DDR) is activated during the initiation of tissue regeneration (A) Schematic representation of the general cellular events taking place during the first week post-amputation (hpa) of the anterior region in planaria (color coded boxes). During the generic wound response, a localized apoptotic response occurs ~4 hpa, accompanied by a systemic burst of mitotic neoblasts at 6 hpa that gradually reduces ~12 hpa. Next, the regeneration response is followed by a localized increase in cell proliferation and a systemic wave of death. After 72 hpa the tissue will continue restructuring newly formed tissue through differentiation of stem cells and progenitors. (B) Waves of gene expression found in the generic wound response within the first 12 hpa. (C) Heat map representing GO term analysis of wound induced DDR gene expression derived from Wenemoser et al. [79]. (D) Percent of DNA damage genes associated within each wave. (E) Temporal wound-induced gene expression waves. Solid lines indicate averages of DDR specific wave response and dotted lines indicate waves according to Wenemoser et al. [79]. (F) Heat map of average DDR gene expression post amputation. At 48 hpa the average expression of all DDR genes is upregulated following wounding. (G) Graph depicts the temporal model of planaria regeneration including the average expression for genes involved in the DDR. The average of all DNA damage gene expression was obtained from the heat map in (F), (blue line) is plotted against the established temporal model of regeneration (dotted lines) according to Wenemoser et al. [79] and is color coded as shown in (A).

narians [142]. It is tantalizing to speculate that the increasing load of genomic instability may act as a switch for zeta neoblasts to leave their lineage-restricted state and try to fill the niche left behind

by sigma cells that cannot survive increasing DNA damage. It is also possible that persistent demands of cellular turnover override fate decisions to promote exit of cell cycle arrest has been noted

in hematopoietic SCs [63]. Some of these cells may have weaker sensors and effectors of the DDR, allowing them to more easily circumvent these checks and balances and ultimately evolve into cancer-like cells. Additional experiments are required to dissect the actual mechanisms driving cells to withstand excessive DNA damage and continue to proliferate. Nonetheless, the results obtained with *Rad51* and *Ubc9* downregulation supports the notion that cellular decisions in the presence of DNA damage are also influenced by regional signals that may involve crosstalk among tissues and organs. This is an important finding as a more comprehensive focus on the regional signals driving proliferation of cells with genomic instability may help in understand the mechanisms facilitating cancer formation and progression.

## 5.2. DNA damage and repair are essential components of the regenerative response

Cell death and proliferation are not only instrumental during tissue renewal but also in the process of regenerating missing or injured body parts. Upon amputation, planaria undergo an orchestrated series of localized and systemic cascades of cellular proliferation and programmed cell death (e.g. ~4–6 h and ~48 h). Recent research have greatly furthered our understanding of the genetic and molecular cascades required for tissue repair and regeneration [79,80,143]. The initial peaks of systemic cell division and localized cell death events were found to be accompanied by a genetic response called the generic wound response that happen during the first 24 h post-injury/amputation (hpa). This is followed by a wave of specific gene expression representing the regeneration response (24–70 hpa) that includes the second molecular peak of mitosis and apoptosis. Finally, the differentiation phase is attributed to neoblast progeny mediated differentiation and specialization of the blastema at +70 hpa [79,80] (Fig. 6A).

The ability to adjust cell proliferation during simultaneous demands of tissue renewal and injury highlight the faithful mechanisms used by planaria to regulate cell number. Since injury repair relies on cell proliferation and consequently DNA replication, we argue that the DDR is an active player that preserves genome integrity during regeneration. In other words, an increase in cell division is accompanied by DNA replication that is carefully monitored by DNA repair mechanisms. Indeed, recent studies have demonstrated that key components of DNA replication and repair (e.g. *p53*, *Rb*, *Rad51* and *Ubc9*) are critical for the regenerative process and without them; planaria fail to regenerate [23,84,86,116]. In addition, cell death is necessary for proper regeneration. The TUNEL assay, which detects cellular apoptosis induced by DSB-nicked ends, is commonly used to evaluate cell death in planaria [105]. After amputation, two peaks of cell death are known to happen at ~4hrs and ~48 h. However, it remains unclear whether these stereotypical patterns of TUNEL + cells are derived from the stressful environment of regeneration or actual DNA damage, specifically DSBs.

We were prompted to reanalyze the possible role DNA damage response play during the early and late phases of tissue regeneration based on published transcriptomic data [79,80]. Transcriptomic changes during the first half of the generic response in planaria offer an interesting resource to discern the role of DDR [79]. This process involves four waves of gene expression found within differentiated tissues (waves 1–3) and neoblasts (wave 4) (Fig. 6B). Using published RNAseq data from Wenemoser et al. [79], we were able to identify genes involved in DNA damage response by GO term analysis. We found that all waves except wave 3 contained genes involved in DDR (Fig. 6C–E). Interestingly, we found PARP-3 as the only DDR gene involved in wave 1 and the only DDR-specific gene with a peak in gene expression within the first hour post amputation. At the decline of PARP-3 expression at 3 h, both wave 2 and

wave 4 DDR specific gene expression increased and peaked at 6 h, which coincide with the system wide mitotic response (Fig. 6C). These results suggest that expression of genes in the DDR follow similar transcriptomic changes of the generic wound response except in wave 3. It also implies that PARP family genes may prime the DDR during early regenerative events (Fig. 6C–E). However, the question still remains whether the first peak of TUNEL + cells starting ~4hrs is linked to DNA damage.

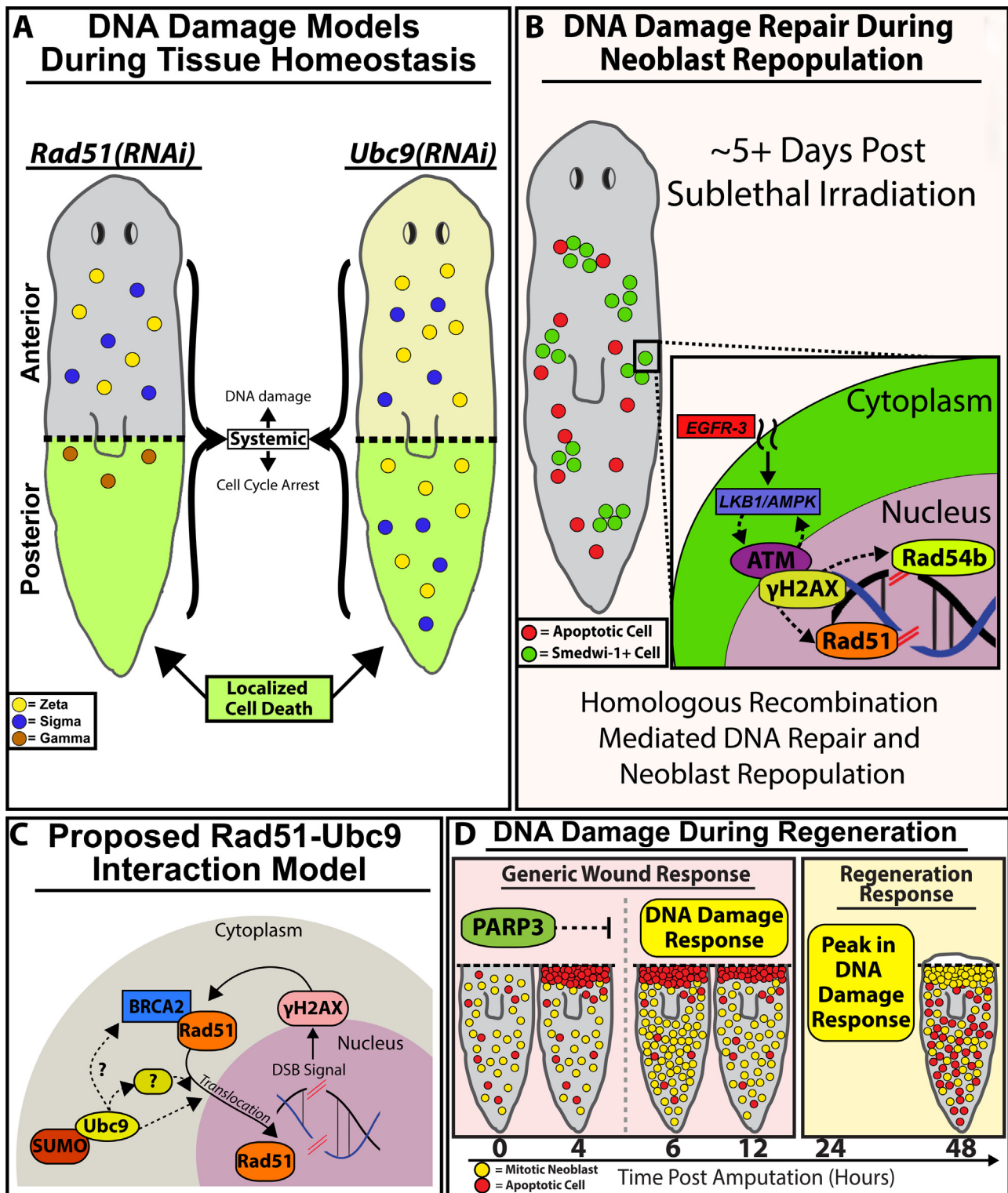
PARP-3 catalyzes post-translational modifications of proteins involved in transcription silencing, cell death and interacts with PARP-1 during DDR to accelerate NHEJ [144–147]. In addition, PARP-3 has been shown to act independently of DNA damage and mediate centrosome stability, G1/S cell cycle progression and telomerase activity [145,148,149]. For instance, PARP-3 expression has an inverse relationship with telomerase activity. Lung cancer cells depleted of PARP-3 displayed an increase in telomerase activity [149]. During planaria regeneration, telomerase activity is upregulated and accompanies neoblast proliferation [150]. Thus, the possible role of PARP-3 expression during the first 3 h of regeneration may be to restrict both neoblast cell cycle progression and telomerase activity. The decline in PARP-3 after 3 h may allow the priming of transcription of both cell cycle and DNA repair proteins that are involved during initial mitotic burst at 6 h. The data analysis shows that the initial localized peak of TUNEL + cells may not be in response to DNA damage but to the harsh environment at the amputation site to facilitate wound closure and there is a possibility that PARP-3 may be mediating the post-translational modifications associated with cell death.

Regenerating planaria exhibit a localized mitotic peak and a systemic increase in TUNEL + cells resulting in the formation of a regenerative blastema starting at 48 h post amputation [79,81,105]. Gene expression levels associated with DNA damage response and repair are relatively low within the first 24 h of regeneration (Fig. 6F). Strikingly, at 48 h, there was a dramatic increase in the expression of genes involved in DNA damage signal transduction and DDR (Fig. 6F). Thus, by plotting the average of DDR gene expression values onto the established temporal model of planaria regeneration [151], we observed that the second wave of DDR is much more significant than that of the generic phase and remains a key feature in the regeneration and early differentiation phases (Fig. 6G). Consistently, our recent work strongly supports the idea that DDR is required for large-scale tissue regeneration. Without key molecules such as *Rad51*, *BRCA2*, *Ubc9* animals, fail to regenerate and replace lost tissues [23,116]. Furthermore, according to Wurtzel et al. [80]; expression levels of genes involved in HR (e.g. *Rad51-A*, *B*, *C* and *BRCA2*) were highly active compared to the decrease in NHEJ (e.g. *Ku70*, *Ku80*, and *Ligase IV*) related genes. However, gene expression analysis revealed that HR and NHEJ were at their peaks at 48 h post amputation. Delving into the molecular dynamics of mitotic events during regeneration in *Rad51* and *Ubc9(RNAi)* animals, we observe that at 6 h post-amputation both groups respond with a slight increase in mitoses but fail to elicit a second mitotic peak at 48 h [23,116]. These results together provide further evidence that DDR is a crucial component of the overall regenerative response in planaria.

## 6. Final remarks

Preserving genomic integrity over recurrent tissue renewal is an important challenge that wanes with age. DNA damage and the cellular responses associated with it are at the center of efficient cellular turnover. Thus, we advocate for studies of SCs in their natural environment as they attend demands of tissue renewal and repair. The analyses integrating SCs, DNA damage and tissue





**Fig. 7.** DNA damage repair (DDR) pathways are critical components of the cell proliferation required for tissue regeneration. (A) *Rad51(RNAi)* (gray) and *Ubc9(RNAi)* (light-yellow) planaria share similar increases in DNA damage along the AP axis accompanied by varying levels of cell cycle arrest. Localized cell death (green) is observed in the posterior region of both RNAi conditions. Survival of neoblasts/sub-types were determined from gene expression data from Fig. 5G; neoblast subtypes: zeta (yellow), sigma (blue) and gamma (brown). (B) 5 days post sublethal irradiation, remaining neoblasts will repair DSBs by homologous recombination and neoblasts will repopulate the entire animal. We propose a hypothetical model whereby EGFR signaling integrates with DDR to facilitate neoblast repopulation post-irradiation (indicated with dotted lines). (C) Proposed RAD51-UBC9 interaction model. Presence of DNA DSB breaks triggers upregulation of RAD51 and γ-H2AX that together participate in DNA repair. However, after *Ubc9(RNAi)* the RAD51 protein accumulates in the cytoplasm as SUMOylation is required for RAD51 translocation to the nucleus. We propose that *Ubc9* may interact with intermediate regulators of Rad51 for DDR. (D) Model of DNA damage during large-scale tissue regeneration. PARP3 expression peaks during the first 4 hpa and may be suppressing DDR gene expression within that timeframe. Upon the decline of PARP3 expression, DDR expression at +6 hpa is observed. A substantial peak in DDR observed in parallel with the regeneration responses that begins +24 hpa. The robust responses of DDR pathways during wound repair could be necessary to drive DNA repair during neoblast proliferation that is required in tissue regeneration.



renewal in the complexity of the whole body present fresh opportunities to the field and has the potential to inform about cellular crosstalk and regulation of signaling pathways (e.g. tumor suppressors, oncogenes) that control cellular decisions in the face of DNA damage (Fig. 7A). The evolutionary and functional conservation of the DDR and mechanisms of SC function in planaria offers a simplified paradigm, in which pharmacological or genetic screens can be performed rapidly and cost-effectively.

The capacity to induce different levels/types of DNA damage with pharmacological compounds, IR and genetic manipulations paves the way for additional studies aimed at understanding the biology of the DDR and possible alternatives for therapeutic applications (Fig. 7B). The genetic models using loss of function of *Rad51* and *Ubc9* enable *in situ* analysis of tissue renewal in response to DNA damage that uniquely allow us to address the molecular basis controlling regional differences in the adult body (Fig. 7A). Furthermore, the activation of regional cell survival and death in response to the systemic presence of DSBs is a great resource to address mechanisms of SC survival with defective DNA. This example prompts the possibility of studying intrinsic and extrinsic cues that may favor or restrict the growth of cancer initiating cells (Fig. 7C, D). What are the signals facilitating survival of SCs with DSB in the anterior, whereas in the posterior surveillance mechanisms remain active and effectively eliminate damaged cells? Our results suggest that neural inputs may influence dynamics of cell cycle in neoblasts carrying DSBs. Thus, future experiments will address the source and the molecules mediating nervous signals that facilitate cell survival in an environment of genomic instability. Since neoblasts in the anterior region tend to survive with DSBs, it brings interesting opportunities to dissect potential differences among SCs and their susceptibility and advantages to DNA damage. Likewise, future studies will also integrate positional cues along the AP axis [152,153] with DNA repair to inform about potential interactions that can enhance or reduce the integrity of the genome. Answering these fundamental questions will enable the field to identify early markers of cellular transformation, mechanisms regulating tumor suppressors, and to define means by which transformed cells survive and form tumors. Finally, we believe that planaria traditionally recognized as model for tissue regeneration, may also represent a fresh alternative to understand and manipulate DNA damage and its effects in the adult body.

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## Competing interest

The authors declare no competing or financial interest.

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